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14. ABSTRACT

Rhodium-105 is an attractive nuclide for radiotherapeutic applications due to its nuclear properties (566 keV β^-, γ = 319 keV [19%], 306 keV [5%]) and the kinetic stability of Rhodium (III) complexes with soft sulfur donor atoms. Extension of previous research involving tetrathioether chelate systems to include a targeting molecule may have implications for prostate cancer therapy. This work reports on the synthesis and evaluation of a new bombesin peptide targeted Rh (III) tetrathioether analogue, [Rh-S4-8Aoc-BBN(7-14)NH$_2$]$^+$, which shows high affinity for the BB2r receptor on PC-3 cancer cells (IC$_{50}$ = 2.2 ± 0.3 nM). However, multiple ^{105}Rh labeled species were obtained under the radiolabeling conditions investigated. To better understand the results observed for [^{105}Rh-8Aoc-BBN(7-14)NH$_2$]$^+$, the chemistries of previously investigated [Rh-S4-Diol]$^+$ and [Rh-S4-(COOH)$_2$]$^+$ were re-evaluated using more recently available techniques. A quantitative evaluation of the [Rh-S4-Diol]$^+$ and [Rh-S4-(COOH)$_2$]$^+$ systems using NMR, ESI-MS and HPLC reveals formation of multiple species resulting from both exchange of the coordinated chlorides at the metal center and esterification of pendant carboxylate groups. While a predominate *trans*-chloro Rh(III)-S4 species may be favored by addition of excess NaCl, both ethanol and acid are required for radiolabeling. Thus, ligand systems utilizing pendant carboxylate groups are not compatible with traditional ^{105}Rh radiolabeling techniques. Future studies involving a ^{105}Rh tetrathioether bombesin analogue without pendant carboxylate groups are recommended.

15. SUBJECT TERMS
Rhodium, Bombesin, Tetrathioether, Radiopharmaceutical, Targeted Radiotherapy

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Introduction:

Since this grant was ended early (June 2013) the annual and final summaries were due at the same time. Thus no additional work was done between the time period covered in the annual report and the final report. The PI completed her Ph.D. in Chemistry at the University of Missouri and has accepted a position with Dr. David Wilson at University of California San Francisco, continuing her career as a translational scientist in the field of prostate cancer research.

Rhodium-105 is an interesting candidate for radiopharmaceutical use due to its nuclear emissions ($\beta^- = 566$ keV; $\gamma = 319$ keV [19%], 306 keV [5%]) and kinetic stability. The goal of this project was to develop a new bombesin (BBN) targeted radiotherapeutic agent for ^{105}Rh using the bifunctional chelate technique. In pursuit of this objective, we have synthesized a new tetrathioether bombesin molecule, S4-8Aoc-BBN(7-14)NH$_2$, which may have implications for prostate cancer therapy. We have also investigated the use of cyclic and acyclic tetrathioether chelates with ^{105}Rh and the implications this chelate choice may have on radiopharmaceutical development. This work adds to the current knowledge of ^{105}Rh ligand systems and will aid future researchers in the selection criteria for viable chelate systems.

Body:

Specific Aim 1: Synthesize bombesin (7-14) coupled tetrathioether bifunctional chelate

The dicarboxylic acid functionalized ligand 3,3,3-S4-(COOH)$_2$ was synthesized according to previously reported methods,[4,5] as shown in Figure 1. Methylthioglycolate (4.0 mL, 42 mmol) and an excess of 1,3-dichloropropane (40 mL, 410 mmol) were brought to reflux at 84°C under nitrogen followed by the dropwise addition of dry triethylamine (TEA, 6.6 mL, 47 mmol). The triethylammonium chloride precipitate was removed by filtration and excess dichloropropane was removed under vacuum at 60°C yielding a yellow oil. The separation of intermediate **1** using the vacuum distillation technique described in Goswami et al[4,5] did not result in sufficient purification. The resulting oil was further purified by silica gel column chromatography with a dry mass of silica gel to sample ratio of 80:1. The

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sample was loaded in a 50% mixture of hexanes and diethyl ether and then eluted with the same solution. The loaded solution was yellow in color. As the yellow band began to elute, fractions were collected and monitored by silica TLC developed in 50% hexanes/diethyl ether. The fractions containing intermediate **1** (Rf = 0.56) were collected and the mobile phase was removed under nitrogen gas. The purified intermediate **1,** recovered in 71% yield, was characterized by ^{1}H NMR spectroscopy. ^{1}H NMR (300 MHz, CDCl$_3$): $\square$ ppm: 3.75 (s, -O-**CH$_3$**, 3H), 3.65 (t, -**CH$_2$**-Cl, 2H), 3.24 (s, -**CH$_2$**-COO, 2H), 2.80 (t, -CH$_2$-**CH$_2$**-S, 2H), 2.07 (p, CH$_2$-**CH$_2$**-CH$_2$, 2H).
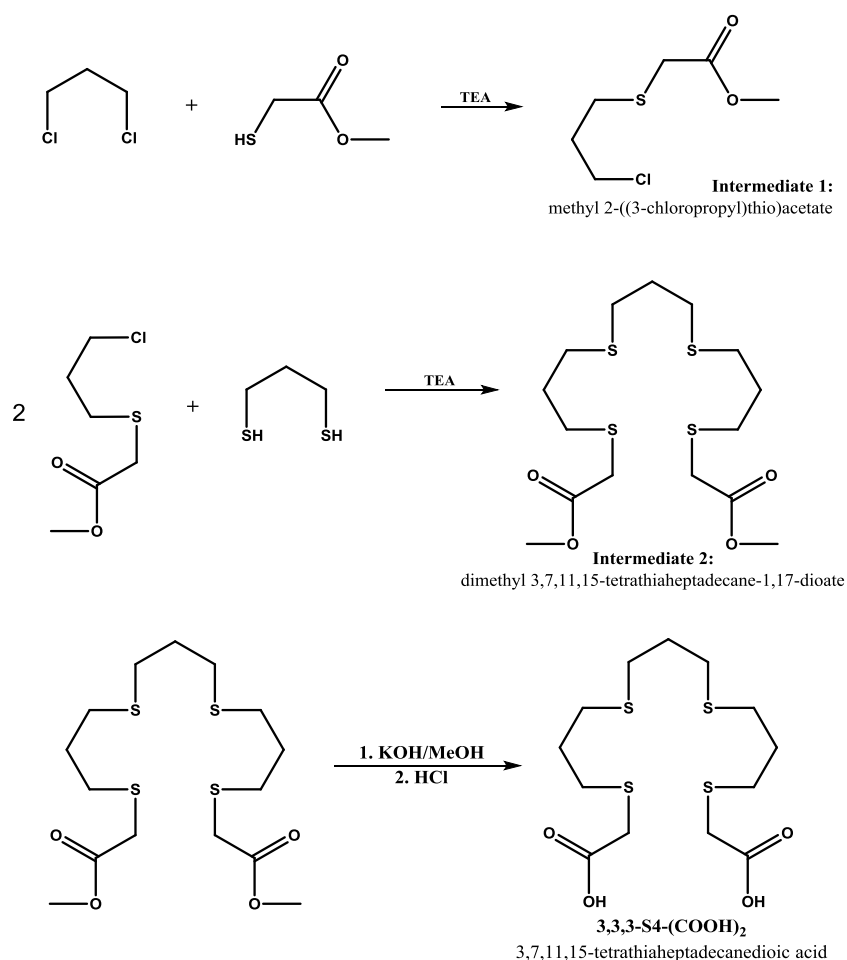

Figure 1: Synthesis of 3,3,3-S4-(COOH)$_2$.

Two molar equivalents of intermediate **1** (3.58 g, 19.6 mmol) were combined with 2.8 mL (20 mmol) of dry triethylamine (TEA). 1,3-propane dithiol (0.96 mL, 9.56 mmol) was added to the mixture

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dropwise, and the solution was refluxed under nitrogen at 94°C for 17 hours. The mixture was then cooled and slurried with ~5 mL of diethyl ether. Triethyl ammonium chloride was removed by filtration and diethyl ether removed under nitrogen. The intermediate **2** oil was purified by silica gel chromatography as described above. A yield of 60% was isolated, ^{1}H NMR (500 MHz, CDCl$_3$): $\square$ ppm: 3.75 (s, -O-C**H**$_3$, 6H), 3.24 (s, -C**H**$_2$-COO, 4H), 2.75 (t, -C**H**$_2$-S-CH$_2$-COO, 4H), 2.62 (t, C**H**$_2$-S-C**H**$_2$, 8H), 1.87 (m, CH$_2$-C**H**$_2$-CH$_2$, 6H).

Intermediate **2** (2.046 g, 5.1 mmol) was dissolved in an alkaline solution of KOH (1.194 g) in 6 mL of methanol and heated to 90°C for 55 min. The solid product was filtered, washed with methanol and dissolved in 10 mL of water. Hydrochloric acid (6 M) was added to the solution dropwise resulting in the precipitation of the desired product 3,3,3-S4-(COOH)$_2$ at pH 2. The precipitate was filtered and dried under vacuum. A 71% yield of the final product was isolated, ^{1}H NMR (500 MHz, CD$_3$CN): $\square$ ppm: 3.22 (s, -C**H**$_2$-COO, 4H), 2.69 (t, -C**H**$_2$-S-CH$_2$-COO, 4H), 2.58 (t, C**H**$_2$-S-C**H**$_2$, 8H), 1.81 (m, C**H**$_2$-S-C**H**$_2$, 6H)

8-Aoc-BBN(7-14)NH$_2$ was synthesized on a solid rink amide resin support using Fmoc protected amino acids. The resin bound amino acids were deprotected with a 0.1 M solution of hydroxybenzoltriazole (HOBT) and 20% piperdine. Then the carboxyl groups were activated with 0.5 M of o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) in DMF, and a mixture of 34.8 mL (0.2 mol) diisoproplyelthyamine (DIEA) in 65.2 mL (0.7 mol) N-methyl-2-pyrrolidone (NMP). The activated end of the growing resin-bound peptide was flushed with an excess of Fmoc-amino acid resulting in the formation of an amide bond.

Manual coupling was used to conjugate the tetrathioether chelate by adding 60 μmol of Fmoc-8Aoc-BBN(7-14)NH$_2$ on the resin support to a stirring solution containing DIEA (50 μL, 300 μmol), NMP (100 μL, 130 μmol), 3,3,3-S4-(COOH)$_2$ (65 mg, 17 μmol), HBTU (45.5 mg, 120 μmol), and HOBT (27 mg, 200 μmol). The solution was stirred at 60°C for 30 min, cooled and then filtered.

S4-8Aoc-BBN(7-14)NH$_2$ was cleaved from the resin support in a solution of 5% water, 5% triisopropyl silane (TIS), and 5% phenol in trifluoroacetic acid (TFA), filtered and precipitated in 12 mL of cold *t*-butyl ether. It should be noted that thiol containing scavengers must be omitted from the cleavage solution as they may react with the chelate thioethers. The desired product was HPLC purified using a Prep Nova-Pak HR C18 Waters column (6 μm, 7.8 x 300 mm, 60 Å) and a binary solvent gradient (80% A, 20% B shifted to 50% A, 50% B over 60 min). The purified product was then analyzed via LC-MS (Figure 2) using a Kromasil C18 HPLC column (5 μm, 150 x 4.6 mm, 100 Å) and a Finnigan TSQ7000 mass spectrometer with the same solvent gradient. (m/z = 1436.15 Da, calc = 1435.68 Da).

RT: 0.41 - 74.98

20% ACN - 50% ACN
over 60 min

NL:
3.43E7
Channel C
UV
303-23V-f5
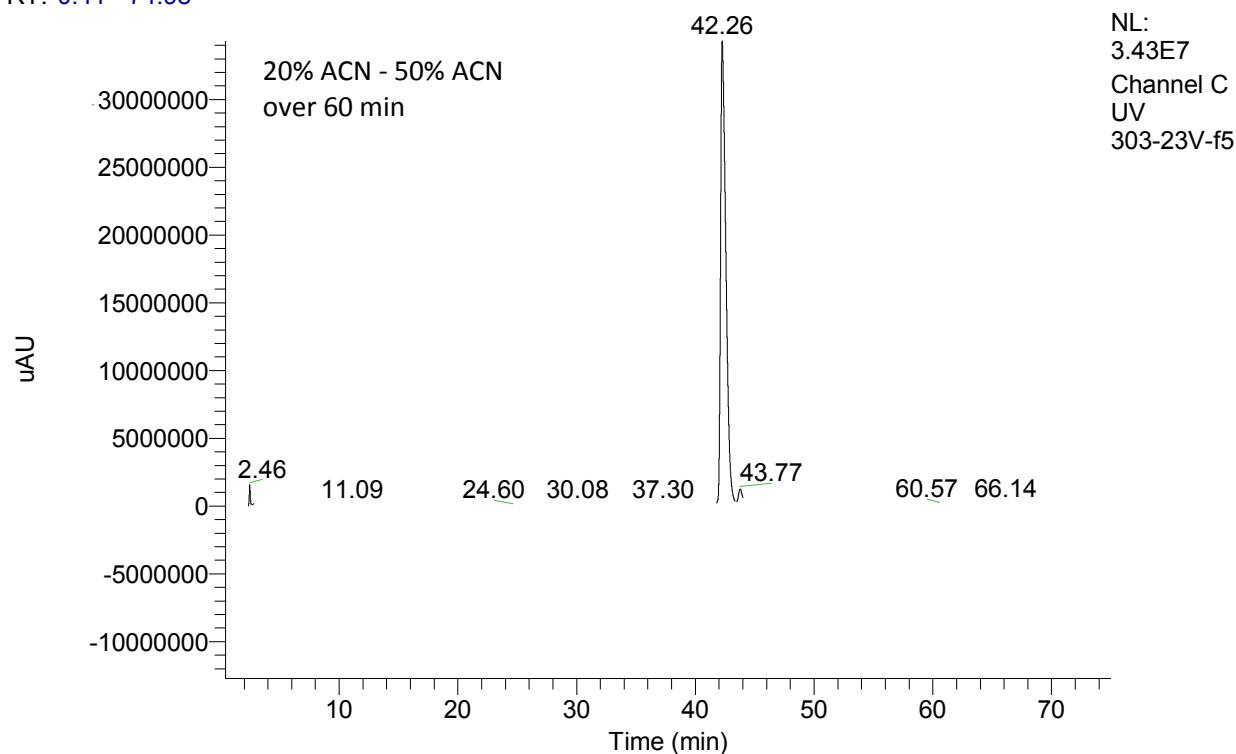
303-23V-f5 #848-862   RT: 42.26-42.96   AV: 15   NL: 4.55E5
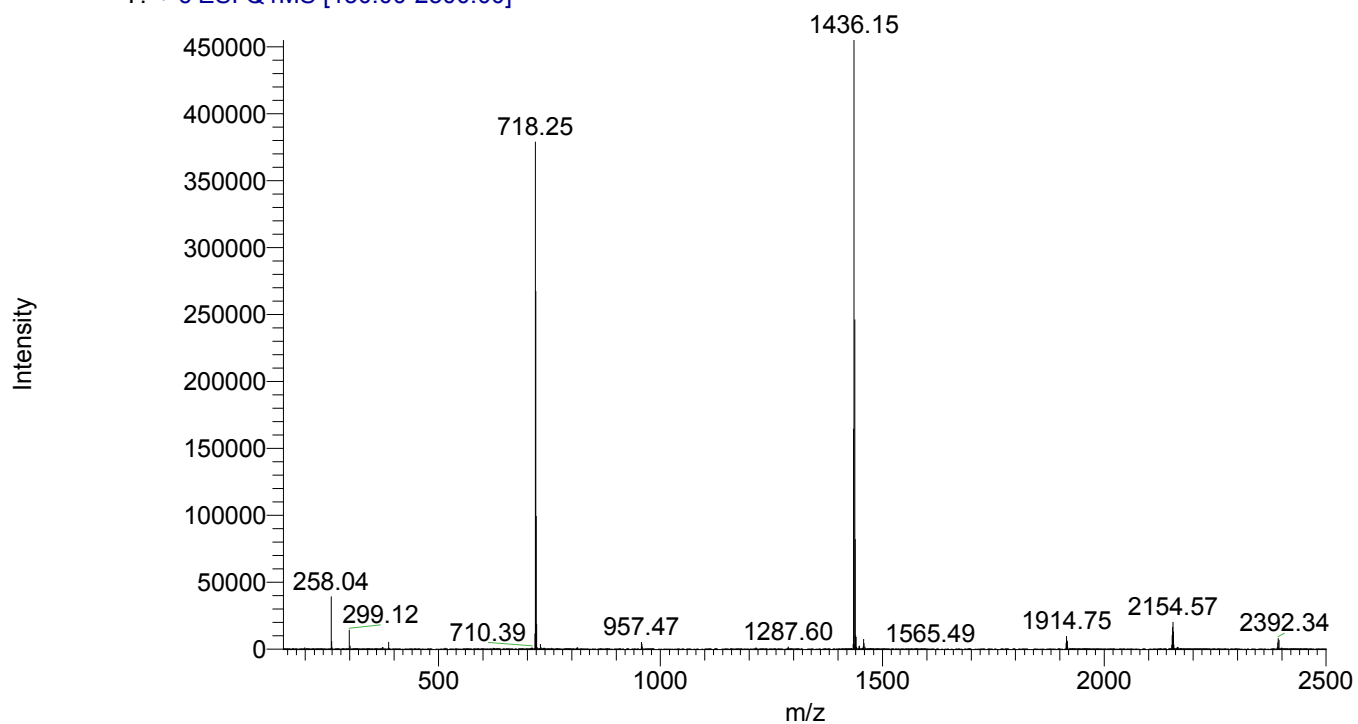T: + c ESI Q1MS [150.00-2500.00]

Figure 2: LC-MS of 3,3,3-S4-8Aoc-BBN(7-14)NH$_2$.

Specific Aim 2: Synthesize and characterize non-radioactive [RhCl-S4-BBN]$^+$

S4-8Aoc-BBN (0.5 mg, 3.1 x 10^{-4} mmol) was dissolved in 5 mL of 4% ethanol/acetonitrile solution. The solution was brought to reflux at 90°C and 40 μL of RhCl$_3$·3H$_2$O (0.9 mg, 3.4 x 10^{-4} mol) in acetonitrile was added dropwise. The mixture was refluxed at 90°C for 1 h, cooled and then lyophilized. The resulting pale yellow solid was analyzed by LC-MS using a Kromasil C18 HPLC column (5 μm, 150 x 4.6 mm, 100 Å) with a Finnigan TSQ7000 mass spectrometer using a solvent gradient of 90% A , 10% B shifted to 50% A, 50% B over a period of 30 min.

LC-MS analysis supports the formation of a [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ complex based on the proposed structure (Figure 3 b) in which the Rh(III) is coordinated to each of the four sulfur atoms, one chloride and a carboxylate pendant group as evidenced by a m/z of 1571.8 Da (calculated = 1571.02 Da) for the [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ and a m/z of 786.3 Da (calculated = 786.51 Da) for the protonated [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^{2+}$ species (Figure 4). This configuration was unexpected based on previous studies with S4-(COOH)$_2$ in which the Rh(III) was coordinated to two chlorides in addition to the four sulfur atoms but not with either of the two pendant carboxylic acid groups (Figure 3 a).[4,5] We expect that the dichloro configuration would dominate in the presence of excess chloride ions in the reaction mixture. This synthesis was done without NaCl added, however during the ^{105}Rh separation significant amounts of NaCl are generated by the addition of HCl to a mixture of NaOCl and NaOH.

The two overlapping LC peaks (Figure 4) at retention times 20.12 min and 20.30 min suggest two chemically similar species. MS analysis of the entire peak area (both 20.12 min and 20.30 min) results in only one m/z value as discussed above, and suggests the presence of a single molecule. As each S is chiral on coordination to the metal center, it is possible that 2 isomers of the target molecule exist. This observation may suggest the presence of two isomers. Since the previous author[4,5] observed only one

trans-dichloro isomer for the 3,3,3-S4-(COOH)$_2$ ligand system, the existence of two isomers would

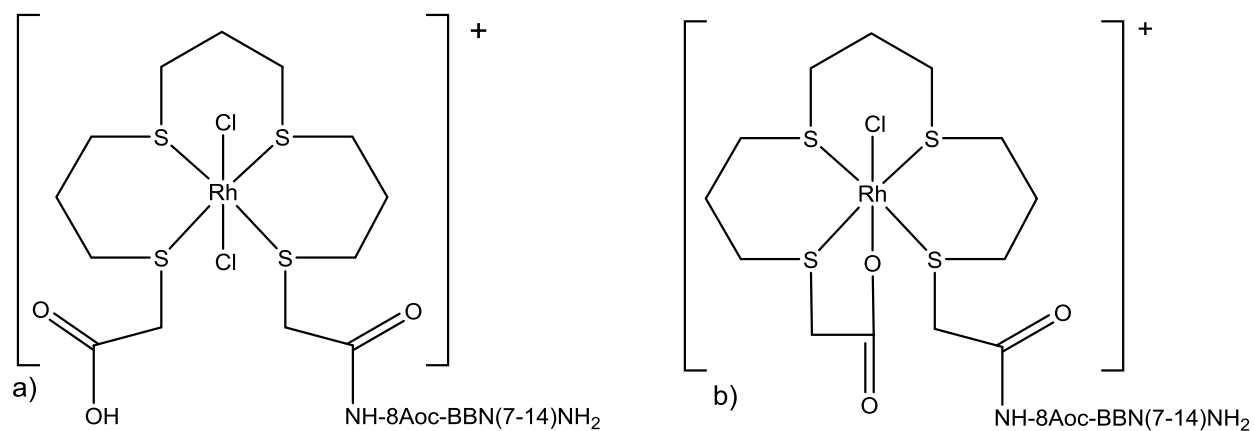further indicate that the expected dichloro species (Figure 3 a) is not present.

Figure 3: Expected configuration of [RhCl$_2$-S4-8Aoc-(7-14)NH$_2$]$^+$ (a)[4,5] and proposed configuration of [RhCl-S4-8Aoc-BB(7-14)NH$_2$]$^+$ (b).

Figure 4: LC-MS of [RhCl-S4-8Aoc-BBN(7-14)]$^+$

Spe   *IC$_{50}$ Evaluation of the non-radioactive [RhCl-S4-8Aoc-BBN]$^+$ complex*

m tetrathioether bombesin, [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ was synthesized during the

firs   project. The previous annual report contains details of chelate synthesis, peptide

coupling and formation of [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$. The affinity of [RhCl-S4-8Aoc-BBN(7-

14)NH$_2$]$^+$ for the gastrin releasing peptide receptor (type BB2) was evaluated using a competitive binding

assay compared to ^{125}I-Tyr[4]-BBN with BB2 receptor positive PC-3 human prostate cancer cells. In a
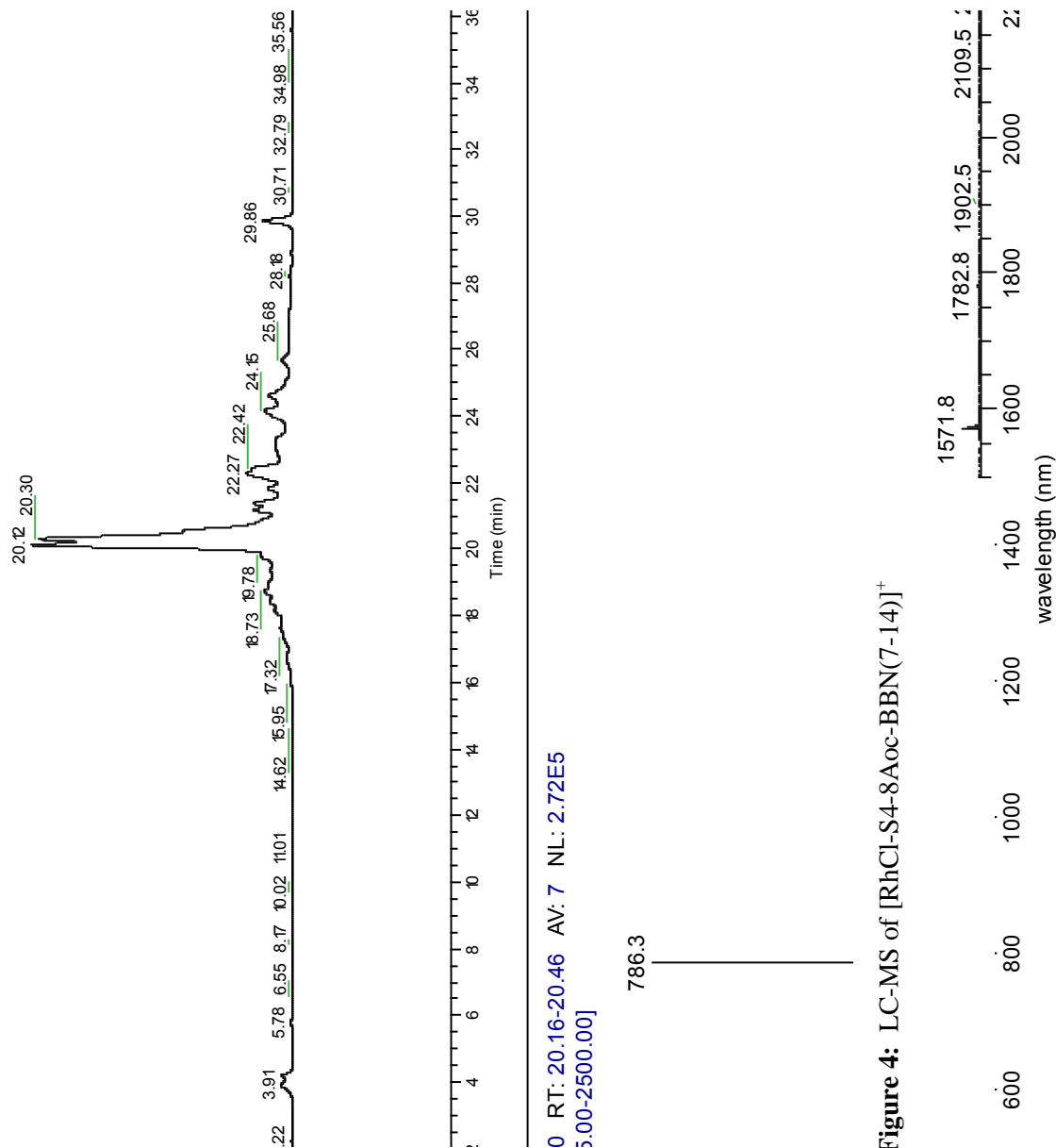
micro-well plate approximately 3×10^5 PC-3 cells were suspended in Roswell Park Memorial Institute (RPMI) medium at pH 7.4 with 4.8 mg/mL HEPES, and 2 mg/mL BSA. The mixture was incubated at 37°C for 1 h with 30,000 cpm of ^{125}I-Tyr-BBN and increasing concentrations of [RhCl-S$_4$-BBN(7-14)NH$_2$]$^+$ from 3.3×10^{-13} to 3.3×10^{-6} M. The cells were washed four times with media to release any non-specifically bound BBN and then counted on a Multi-Wiper (Laboratory Tecnologies, Maple Park, IL, USA) multiwell NaI gamma scintillation detector. The inhibitory concentration 50% (IC$_{50}$) was derived from the average of three experiments. The IC$_{50}$ curve (Figure 5) was obtained by plotting the % of ^{125}I-Tyr-BBN bound to the cell as a function of the concentration of [RhCl-S$_4$-BBN(7-14)NH$_2$]$^+$ added using GraphFit software version 4 (Erithacus Software Limited, Middlesex, UK).

The average concentration of [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ needed to inhibit ^{125}I-Tyr^4-BBN by 50% (IC$_{50}$) was determined to be 2.2 ± 0.3 nM. This value represents a significant improvement over the previously reported macrocyclic Rh-S$_4$- BBN(7-14)NH$_2$ (IC$_{50}$ = 37.5 ± 10.5 nM) and Rh-S$_4$-5-Ava-BBN(7-14)NH$_2$ (IC$_{50}$ = 4.76 ± 0.79 nM)[1,2] and indeed is well within the range of recently investigated pre-clinical bombesin analogues for targeting prostate cancer.
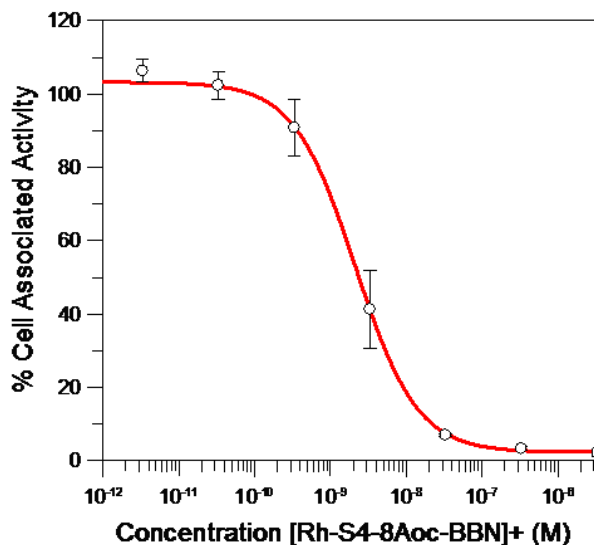

Figure 5: [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ vs ^{125}I-Tyr^4-BBN competitive binding Assay.

*Specific Aim 4: Radiolabeling of the S4-8Aoc-BBN(7-14)NH$_2$ chelate.*

An ethanolic solution of S4-8Aoc-BBN(7-14)NH$_2$ was added to an aqueous solution of 0.5 - 1 mCi of rhodium-105 chloride at pH 3 - 4. Labeling conditions were varied from 2.5% - 57% ethanol and 5.8 x 10^{-5} M - 1.16 x 10^{-3} M S4-8Aoc-BBN(7-14)NH$_2$. In general, the following labeling conditions were investigated;

- 500 µL (~ 1 mCi) of Rh-105, 100 µL of 0.5 mg/mL S4-8Aoc-BBN(7-14)NH$_2$ in 15% ethanol.
  Total ligand concentration: 5.8 x 10^{-5} M, Total ethanol: 2.5%

- 500 µL (~ 1 mCi) of Rh-105, 100 µL of 0.67 mg/mL S4-8Aoc-BBN(7-14)NH$_2$ in 15% ethanol.
  Total ligand concentration: 7.8 x 10^{-5} M, Total ethanol: 2.5%

- 20 µL (~ 50 µCi) of Rh-105, 200 µL of 0.67 mg/mL S4-8Aoc-BBN(7-14)NH$_2$ in 15% ethanol.
  Total ligand concentration: 4.2 x 10^{-4} M, Total ethanol: 13.6%

- 0.05 mg lyophilized S4-8Aoc-BBN(7-14)NH$_2$ in 50 µL acetonitrile, 100 µL ethanol
  200 µL (~ 1 mCi) of Rh-105 stock
  Total ligand concentration: 9.46 x 10^{-4} M, Total ethanol: 28.5%

- 0.067 mg lyophilized S4-8Aoc-BBN(7-14)NH$_2$ in 50 µL acetonitrile, 100 µL ethanol
  100 µL (~ 0.5 mCi) of Rh-105 stock
  Total ligand concentration: 1.84 x 10^{-4} M, Total ethanol: 40%

- 0.067 mg lyophilized S4-8Aoc-BBN(7-14)NH$_2$ in 50 µL acetonitrile, 200 µL ethanol
  100 µL (~ 1 mCi) of Rh-105 stock. (200 µL was concentrated to 100 µL at 90°C, N$_2$)
  Total ligand concentration: 1.33 x 10^{-4} M, Total ethanol: 57.1%

- 0.5 mg lyophilized S4-8Aoc-BBN(7-14)NH$_2$ in 100 µL acetonitrile, 100 µL ethanol
  100 µL (~ 0.5 mCi) of Rh-105 stock
  Total ligand concentration: 1.16 x 10^{-3} M, Total ethanol: 33%

After 1 h of heating at 80°C, all of these conditions resulted in low labeling yields (< 5 - 10%) as measured by analytical HPLC (Figure 6, a). Additional heating (85 ^{o}C, 2 h) resulted in formation of many radiolabeled species also observed by HPLC (Figure 6 b and c). The multiple products likely resulted from both hydrolysis of amide bonds on the peptide due to excess heat and from the existence of multiple [105]Rh-S4 species. For comparison, analytical HPLC chromatograms of non-radioactive S4-

8Aoc-BBN(7-14)NH$_2$ (Figure 7, a) and [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ (Figure 7, b) using the same solvent gradient, column and system are included below. To better understand the results we encountered with radiolabeling [105]Rh-S4-8Aoc-BBN(7-14)NH$_2$, the [105]Rh-S4 chemistry with cyclic 3,3,3,3-S4-Diol[3] and acyclic 3,3,3-S4-(COOH)$_2$[4-6] chelate systems was revisited.

(A) ^{105}Rh-S4-8Aoc-BBN(7-14)NH$_2$ heated 1 h
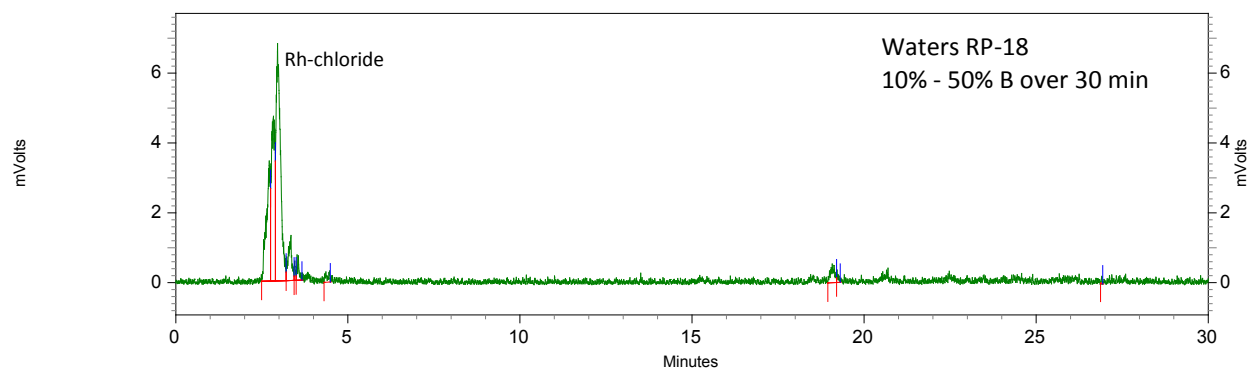
(B) ^{105}Rh-S4-8Aoc-BBN(7-14)NH$_2$ heated 2h
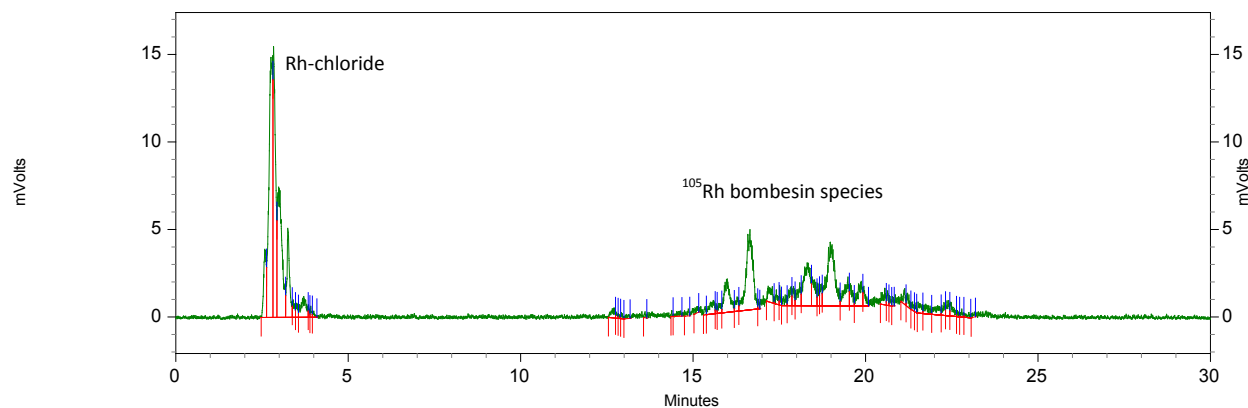
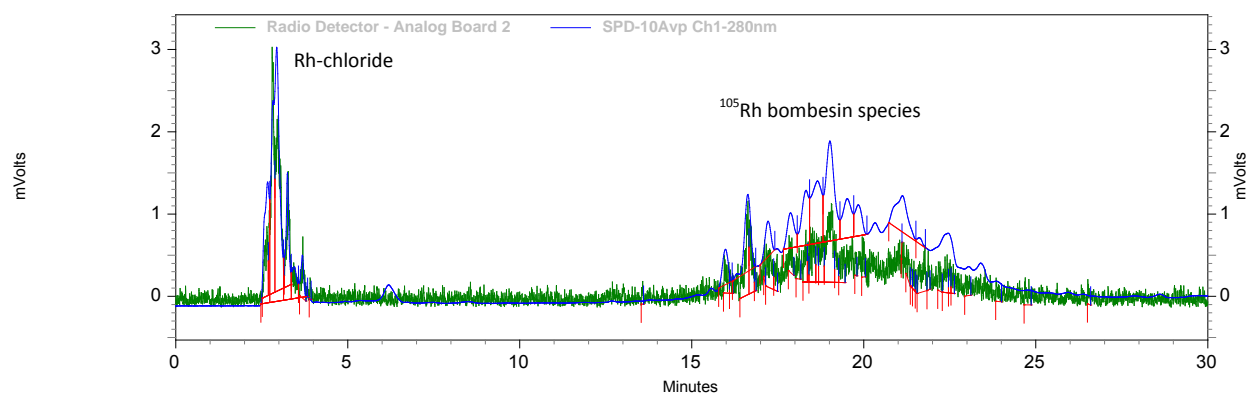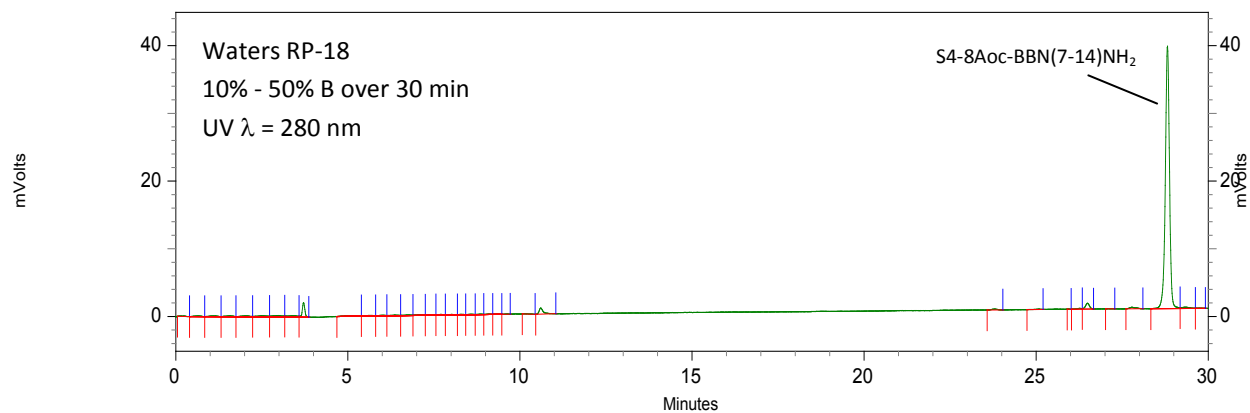(C) ^{105}Rh-S4-8Aoc-BBN(7-14)NH$_2$ heated 2h with cold RhCl$_3$ spike

Figure 6: Representative HPLC radio chromatogram of ^{105}Rh-S4-8Aoc-BBN(7-14)NH$_2$ for (a) 1 h heat at 80°C, (b) 2 h heat at 80°C, and (c) 2h heat with a spike of non-radioactive RhCl$_3$•3H$_2$O.

(A) 3,3,3-S4-8Aoc-BBN(7-14)NH$_2$

Waters RP-18
10% - 50% B over 30 min
UV λ = 280 nm

S4-8Aoc-BBN(7-14)NH$_2$

(B) [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$
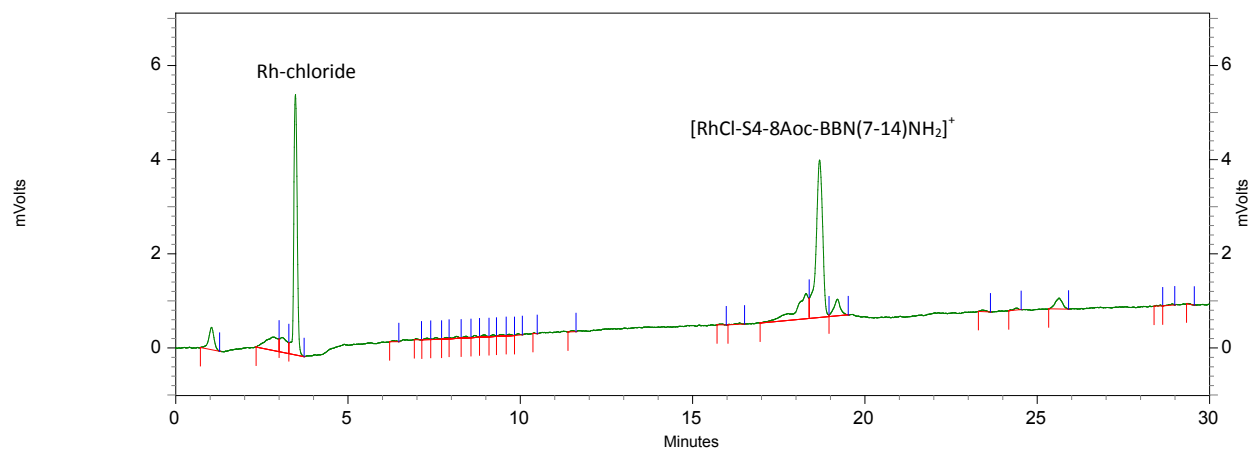

Rh-chloride

[RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$

Figure 7: HPLC chromatogram of (a) 3,3,3-S4-8Aoc-BBN(7-14)NH$_2$ and (b) [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ prepared on a macroscopic scale.

Investigation of the 3,3,3,3-S4-Diol chelate system

The macrocyclic 3,3,3,3-S4-Diol investigated by Venkatesh et al.[3] is a well-known chelate for ^{105}Rh. This complex has been shown to provide >90% yields with ^{105}Rh via a quick labeling procedure and has previously been well characterized by silica gel TLC. Until recently 3,3,3,3-S4-Diol has been available commercially. For this reason many researchers studying new chelate systems for ^{105}Rh have used the ^{105}Rh-S4-Diol labeling procedure and analysis as a quick quality control procedure to determine the labeling efficiency of ^{105}Rhodium Chloride. We have elaborated on the previous TLC evaluation to include an HPLC method of evaluation. This new analysis has provided interesting results pertaining to labeling of thioether complexes with ^{105}Rh.

Non-radioactive RhCl$_2$-S4-Diol was prepared following the procedure reported by Venketesh[3]. Briefly 0.80 mL(3.0×10^{-6} mol) of a 1.0 mg/mL solution of RhCl$_3 \cdot 3$H$_2$O in acetonitrile was added to 1.0 mL (3.0×10^{-6} mol) of a 1.0 mg/mL solution of 3,3,3,3-S4-Diol in either 10% Ethanol/H$_2$O or 10% Ethanol/Saline at pH 4. The solution was heated at 80 $^{\circ}$C for 1 h. Formation of chelated Rh-S4-Diol was confirmed by mass spectrometry. The macroscopic Rh-S4-Diol complex was evaluated by HPLC using a Waters Symmetry Shield RP-18 column (5 μm, 4.6 x 250 mm) with binary gradient where A is increased from 1% to 90% over 8 min, remains linear at 90% until 9 min and is decreased from 90% back to 1% by 10 min.

The radiolabeled ^{105}Rh-S4-Diol was synthesized according to the previously published procedure.[22] Briefly, 100 μL (3.0×10^{-4} mol) of a 0.1 mg/mL solution of 3,3,3-S4-Diol in 15% Ethanol/H$_2$O was added to 500 μL (1 - 2 mCi) of ^{105}Rh Chloride at pH 4 and heated for 1 h at 80 $^{\circ}$C. The resulting solution was spotted on a silica gel TLC plate and developed in 0.9% saline. The labeling solution was also evaluated using the HPLC method described above and compared to macroscopic results.

A 40 µL aliquot of the reaction mixture described above was also spiked with 20 µL (3.8×10^{-5} mol) of cold 1 mg/mL $RhCl_3 \cdot 3H_2O$ and heated for an additional hour at 80 °C. Again this mixture was analyzed using HPLC allowing for in situ confirmation of radio chromatographic peaks with species observed via UV detection.

HPLC analysis of the non-radioactive Rh-S4-Diol complex prepared in a pH 3 aqueous ethanolic solution resulted in a single peak with a retention time of 5.65 min using a gradient of 1% B – 90% B over 8min (Figure 8, c). However analysis of the radiolabeled [105]Rh-S4-Diol exhibited two peaks under the same HPLC conditions, one at 5.81 min and a second peak at 5.53 min (Figure 8, d). A second non-radioactive Rh-S4-Diol complex was prepared in pH 3 ethanolic solution with excess NaCl. HPLC analysis of this solution revealed two peaks at 5.54 min and 5.80 min (Figure 8 e). ESI-MS evaluation (Figure 9) of the macroscopic solutions indicates the presence of both *trans*-$[Rh(OH)_2$-S4-Diol$]^+$ (m/z = 464.92 Da, calc = 464.98 Da) (Figure 9, a) and *trans*-$[RhCl_2$-S4-Diol$]^+$ (m/z = 500.97 Da, calc = 500.91 Da) (Figure 8 b) based on the proposed structures (Figure 10).

This new data provides valuable information about the impact of reaction conditions on the species of *trans*-RhX_2-S4-Diol formed and indicates that the radiotracer chemistry of [105]Rh complexes must be carefully evaluated for multiple isomers when halides are coordinated to the metal center. In the presence of a reducing agent such as ethanol, the coordinated halides are fairly labile and may be exchanged. Additionally, the HPLC method developed represents a new quantitative QC method for future researchers to analyze [105]Rh chloride labeling efficiency.
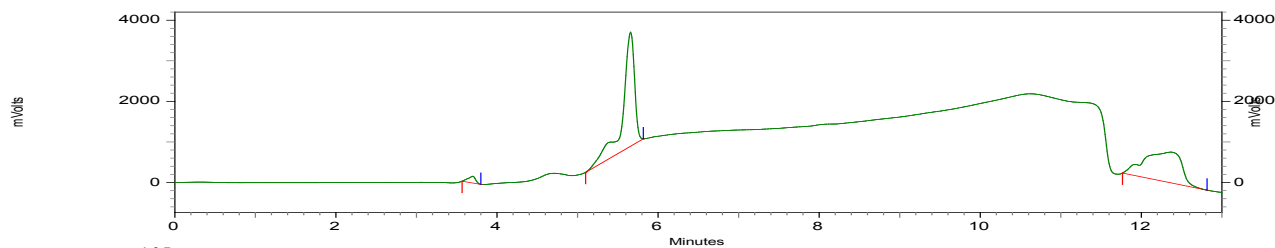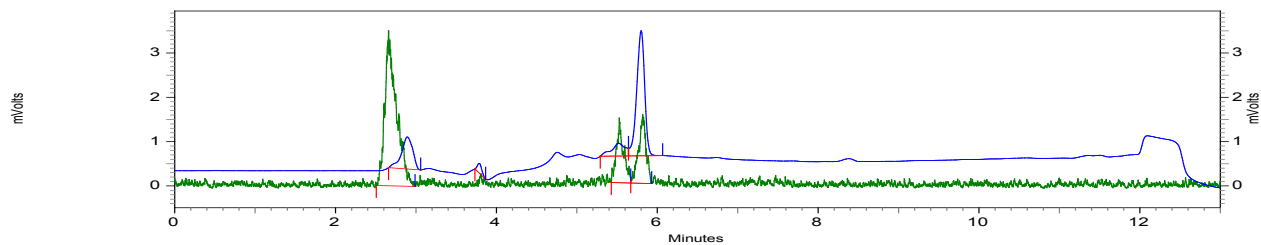
(A) Blank injection
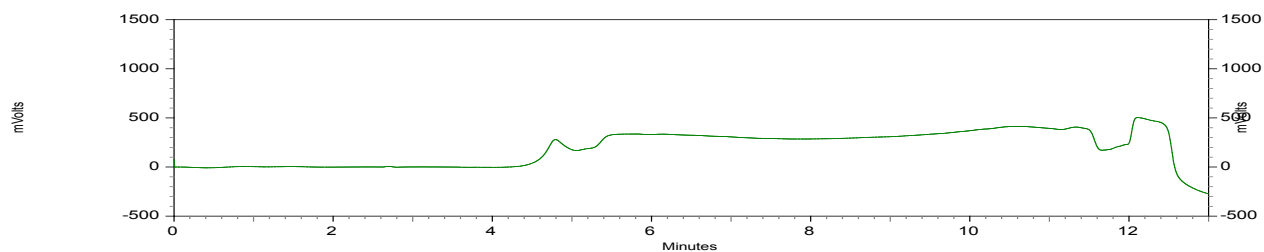
(B) Free Rhodium Chloride
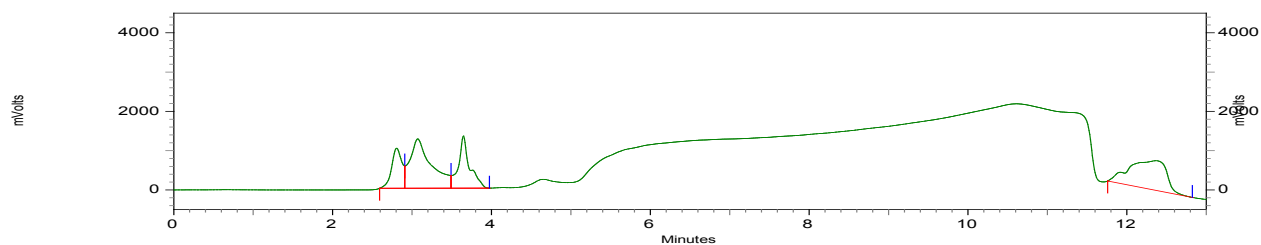
(C) Rh-S4-Diol prepared in water

(D) ^{105}Rh-S4Diol with cold [Rh-S4(OH)$_2$-Diol]$^+$ spike

(E) Rh-S4-Diol prepared in Saline
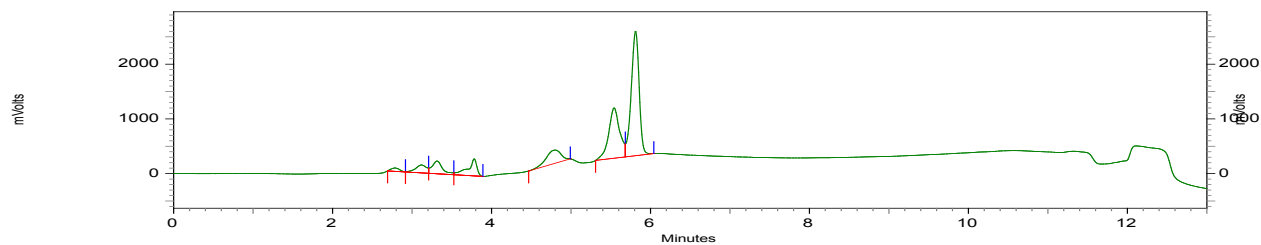

Figure 8: Summary of Rh-S4-Diol HPLC analysis.

19

B #1236-1720   RT: 4.66-6.49   AV: 485   NL: 9.90E3
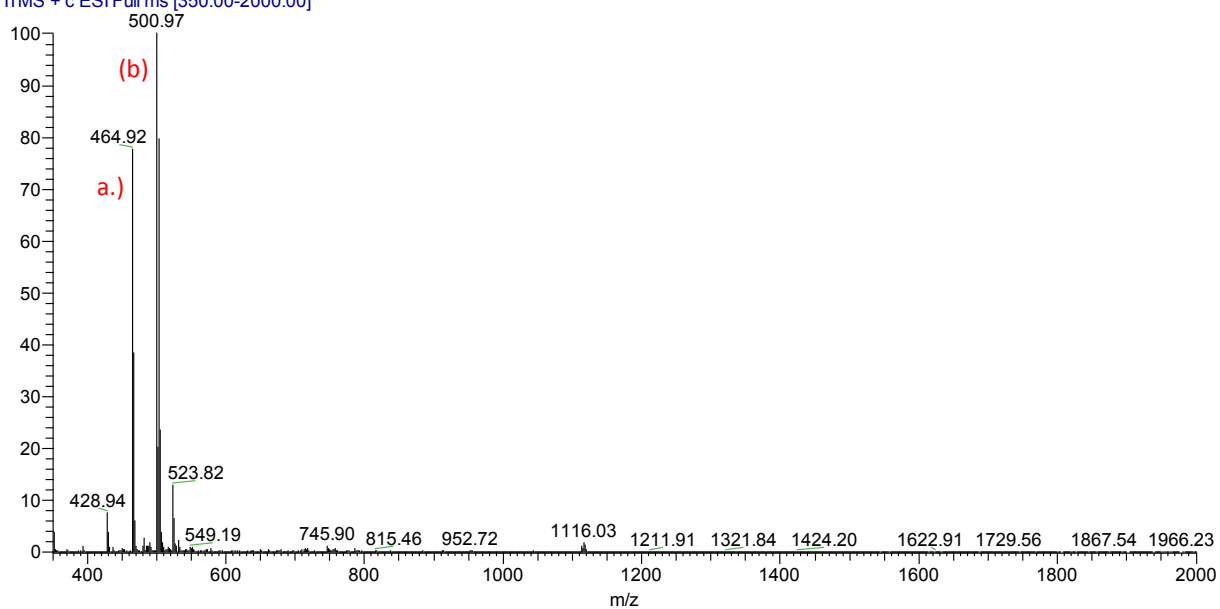T: ITMS + c ESI Full ms [350.00-2000.00]

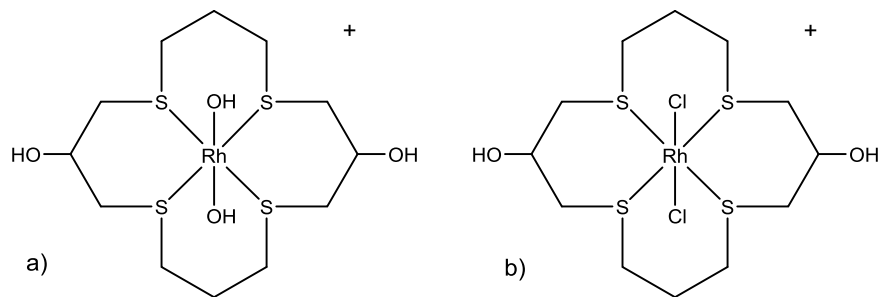**Figure 9:** ESI-MS evaluation of Rh-S4-Diol prepared in saline.

Figure 10: Macroscopic species, Rh(OH)$_2$-S4-Diol (a) and RhCl$_2$-S4-Diol (b).

Investigation of the 3,3,3-S4-(COOH)$_2$ chelate system

The 3,3,3-S4-(COOH)$_2$ ligand system previously studied by Goswami et al.[4-6] is most similar to our S4-8Aoc-BBN(7-14)NH$_2$ molecule. Using 3,3,3-S4-(COOH)$_2$ as a starting material, S4-8Aoc-BBN(7-14)NH$_2$ is formed by coupling the bombesin peptide to one of the carboxylate pendant groups on 3,3,-S4-(COOH)$_2$. Goswami et al. reports formation of a single radiolabeled *trans*-[^{105}RhCl$_2$-S4-(COOH)$_2$]$^+$ species as evaluated by silica gel thin layer chromatography (TLC) in which the product does not move from the origin; only ^{105}Rh chloride moves with the solvent front in saline.[4-6] Based on the results observed for *trans*-RhX$_2$-S4-Diol, it was suspected that a number of Rh-S4-(COOH)$_2$ species were possible for this preparation as well and therefore a more quantitative analysis was performed using HPLC, mass spectrometry and NMR.

Non-radioactive Rh-S4-(COOH)$_2$ was prepared in ethanolic solutions with and without excess NaCl analogous to the Rh-S4-Diol preparation described above and analyzed by the same HPLC procedure. In the absence of excess NaCl the primary Rh(III) species formed is the mono chloride species, [RhCl-S4(COOH)(COO$^-$)]$^+$ with one coordinated pendant carboxylate group (m/z = 508.83 Da, calc = 508.92 Da) (Figure 11, a). This species is observed at an HPLC retention time of 6.14 min (Figure 12, b). When prepared with excess NaCl present the two species observed are *trans*-[RhCl$_2$-S4-(COOH)$_2$]$^+$ (m/z = 544.93 Da, calc = 544.90 Da) (Figure 11, b) with a retention time of 6.87 min (Figure 12, c) and [RhCl-S4(COOH)(COO$^-$)]$^+$ with a retention time of 6.26 min (Figure 12, c). As previously discussed, in the presence of ethanol the coordinated chlorides are readily exchanged. If chloride ions (i.e., salt, NaCl) are not present in sufficient concentration, the coordinated chloride may exchange for another nearby donor atom, in this case a pendant carboxylate.
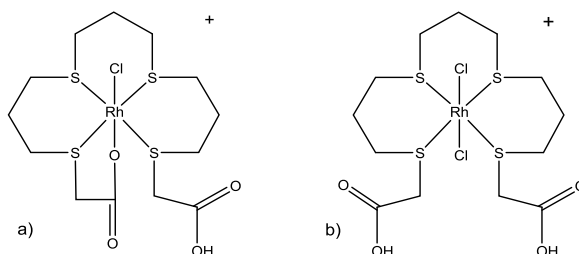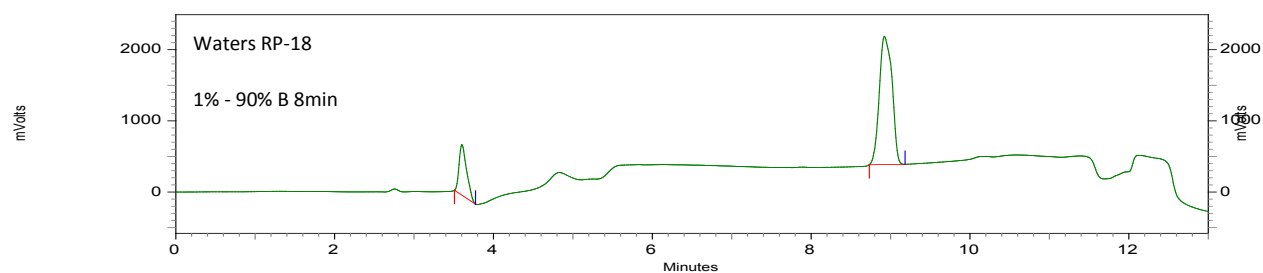

Figure 11: Initial species observed in initial macroscopic preparations.

(A) 3,3,3-S4-(COOH)2 Ligand

Waters RP-18
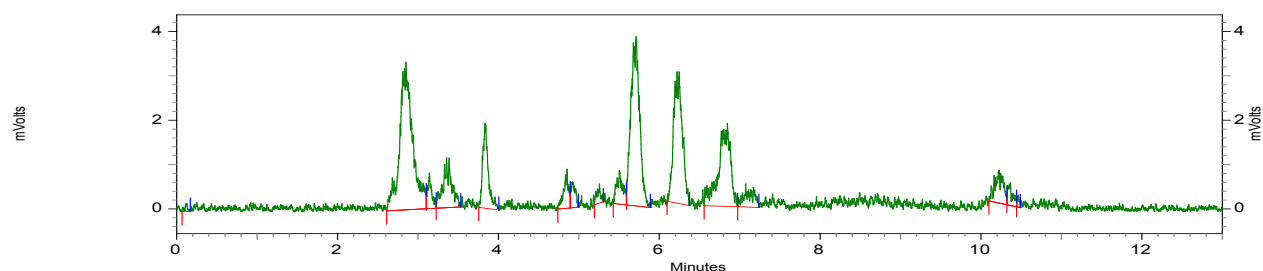
1% - 90% B 8min

(B) RhX$_2$-S4-(COOH)$_2$ prepared no salt
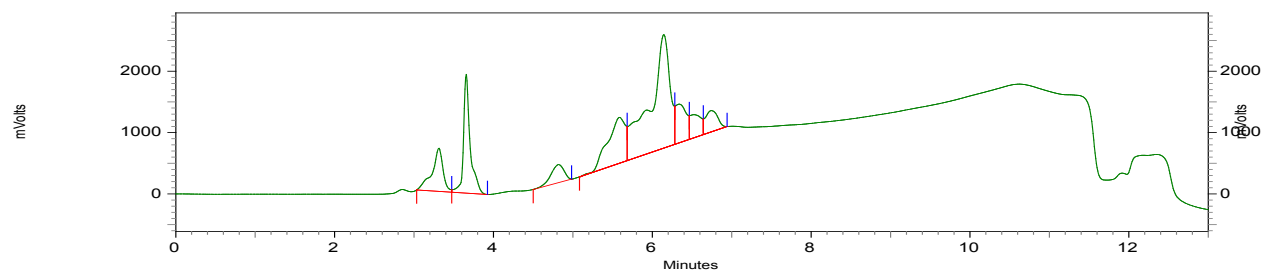
(C) Rh-S4-(COOH)$_2$ prepared in saline
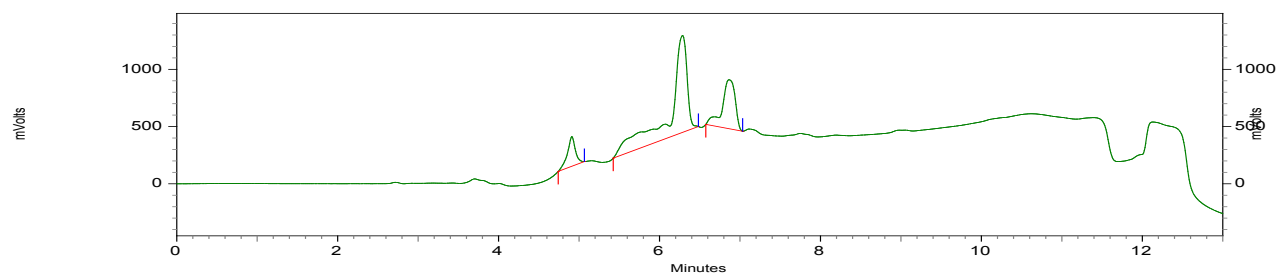
(D) ^{105}RhX$_2$-S4-(COOH)$_2$

(E) ^{105}RhX$_2$-S4-(COOH)$_2$ spiked with cold RhCl$_3$, heated 1h
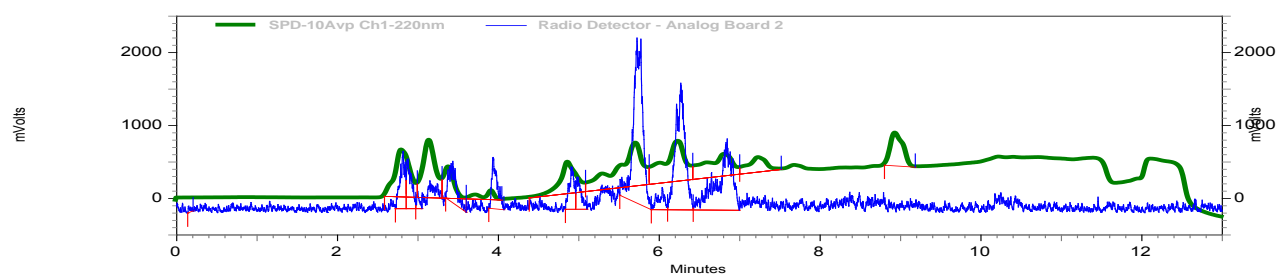
Figure 12: Summary of *trans*-RhX$_2$-S4-(COOH)$_2$ HPLC analysis

Since the existence of at least two *trans*-[RhX$_2$-(COOH)$_2$]$^+$ species has been confirmed on a macroscopic scale, it is necessary to evaluate the radiotracer behavior. The [105]Rh chloride stock solution is in dilute HCl (pH ~ 1) following separation from the [104]Ru target material at the University of Missouri Research Reactor (MURR). Before labeling, this sample is adjusted to pH 3 - 4 with 0.1 M NaOH, which generates NaCl. We hypothesized that radiolabeling of the S4-(COOH)$_2$ ligand under these conditions would result in predominately the *trans*-[RhCl$_2$-S4-(COOH)$_2$]$^+$ species. However HPLC analysis of the radiolabeling reaction mixture revealed at least 4 different peaks with retention times of 4.87 min, 5.71 min, 6.24 min and 6.82 min (Figure 12, d). The radiolabeled mixture was spiked with non-radioactive RhCl$_3$·3H$_2$O and heated for an additional hour. This test generates "carrier" Rh-S4-(COOH)$_2$ compounds in macroscopic amounts, which can be observed by UV ($\lambda = 220$ nm) to confirm a radiochromatographic peak and will highlight any differences between chemistry that occurs on the tracer level and chemistry that occurs on the macroscopic level under the same conditions. The UV trace confirmed all 4 of the tracer peaks observed (Figure 12, e). It is clear that under these conditions it is possible to make multiple Rh-S4-(COOH)$_2$ species. At this point, we hypothesized that the additional peaks by HPLC may be due to any combination of dichloro, dihydroxo, and pendant carboxylate coordinated *trans*-RhX$_2$-S4-(COOH)$_2$ species.

During the initial macroscopic evaluation of *trans*-[RhX$_2$-S4-(COOH)$_2$]$^+$ two species were observed where either X$_2$ = Cl$_2$ ($t_r = 6.87$ min) or X$_2$ = Cl, pendant COO$^-$ ($t_r = 6.14$ min) depending on the concentration of NaCl present in solution. Using the published radiolabeling conditions[4,6] more than two *trans*-[[105]RhX$_2$-S4-(COOH)$_2$]$^+$ species were observed by HPLC ($t_r = 4.87$ min, 5.71 min, 6.24 min and 6.82 min). It was suspected that the *trans*-[Rh(OH)$_2$-S4-(COOH)$_2$]$^+$ species might also be possible in a manner analogous to the observed trans-[Rh(OH)$_2$-S4-Diol]$^+$ when water is present. Therefore reaction conditions varying the amount of water present and the amount of salt present were investigated.

In order to identify as many Rh-S4-(COOH)$_2$ species as possible, the complex was formulated under a series of reaction conditions all at pH 3: (1) all organic solvent reaction conditions (50%

ethanol/acetonitrile), (2) an organic solvent system with excess NaCl (saturated), (3) an aqueous-organic solvent mixture (25% water, 25% ethanol, 50% acetonitrile) and (4) an aqueous solution with excess NaCl (0.1 g). All reaction mixtures were heated for 1 h at 80°C, cooled, and then analyzed using the same HPLC method described above. The reaction mixtures were then lyophilized and taken up in either 50% acetonitrile/water for ESI-MS evaluation or 50% d_3-acetonitrile/D_2O for NMR studies.

HPLC evaluation (Waters RP-18, 1% B – 90% B over 8 min) of sample (1) prepared in 50% ethanol/acetonitrile resulted in a primary peak with a retention time of 6.20 min. Sample (2) prepared in 50% ethanol/acetonitrile saturated with NaCl resulted in a primary peak with a retention time of 6.19 min. The solubility of NaCl in ethanol and acetonitrile is low, 0.65 g/kg and 0.003 g/kg respectively. Thus the conditions in these two preparations are quite similar and similar HPLC results are not surprising.

The macroscopic synthesis of *trans*-[RhCl$_2$-S4-(COOH)$_2$]$^+$ previously reported[4,5] was carried out in acetonitrile/ethanol solution. The radiotracer synthesis is carried out quite differently because of the aqueous starting solution available for [105]Rh-chloride following separation from its target. Our macroscopic preparation of samples (3) prepared in 25% water, 25% ethanol, 50% acetonitrile and (4) prepared in 25% water, 25% ethanol, 50% acetonitrile with 0.1 g NaCl are more similar to radiolabeling conditions.

HPLC evaluation of sample (3) prepared in 25% water, 25% ethanol, 50% acetonitrile resulted in a broad peak (or group of overlapping peaks) with retention times of ~ 5.06 – 6.54 min. Within this region two significant peaks are observed with retention times of 5.68 min and 6.21min. Under these conditions it appears that many species are formed.

Evaluation of sample (4) prepared in 25% water, 25% ethanol, 50% acetonitrile with 0.1 g NaCl resulted in a primary peak with a retention time of 6.18 min. This result suggests that addition of excess NaCl may be used to encourage formation of a single predominate species.

The amount of NaCl present during radiotracer formulation of *trans*-[^{105}RhX$_2$-S4-(COOH)$_2$]$^+$ is currently unknown. During separation of ^{105}Rh from $^{104/105}$Ru, significant amounts of NaCl are generated by the addition of HCl to a mixture of NaOCl and NaOH. The NaOCl was generated *in situ* by bubbling of Cl$_2$ gas into NaOH. Since it is unknown how much NaOCl is generated at this step, the amount of NaCl generated by addition of acid to the separation solution is also unknown. It is possible that the concentration of NaCl present in our radiolabelling formulation is less than the amount present (0.025 mg/mL) in sample (4). Addition of more salt to the radiolabeling solution may improve the yield of a single (or more predominate) species.
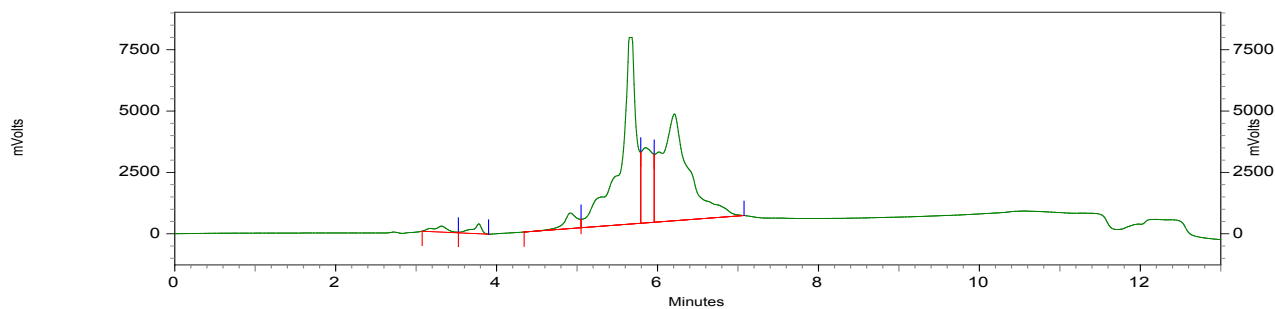
Sample (1) 50% Ethanol/Acetonitrile
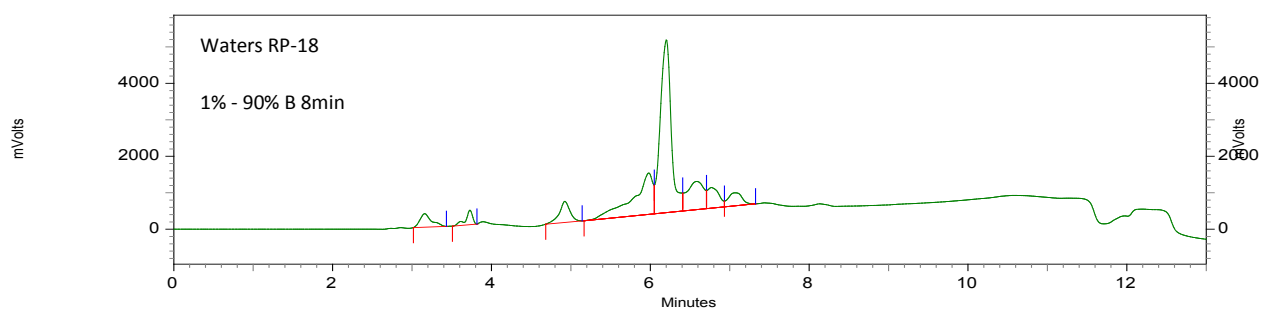
Sample (2) 50% Ethanol/Acetonitrile + NaCl
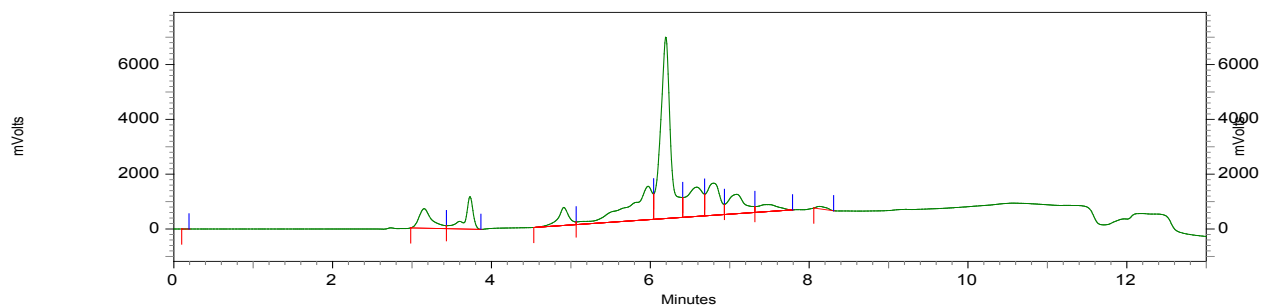
Sample (3) 25% Water/25% Ethanol/50% Acetonitrile

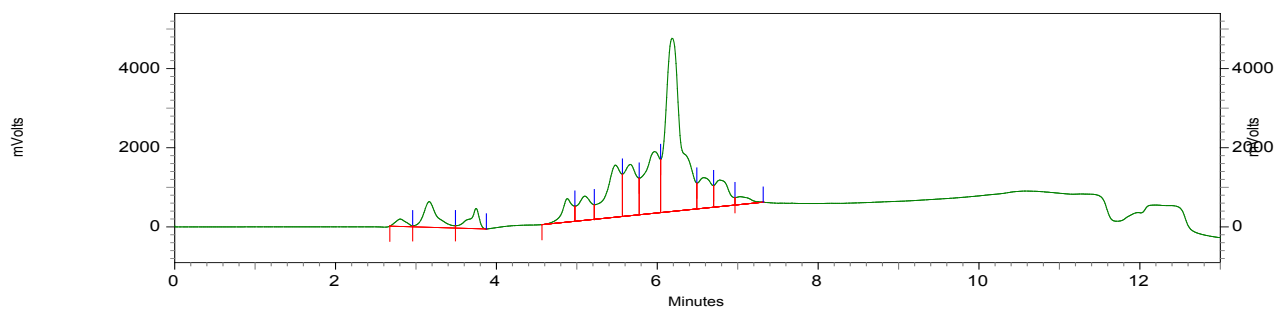Sample (4) 25% Water/25% Ethanol/50% Acetonitrile + NaCl

Figure 13: HPLC analysis of samples prepared under reaction conditions 1-4.

The lyophilized *trans*-[RhX$_2$-S4-(COOH)$_2$]$^+$ was taken up in d$_3$-acetonitrile and evaluated by NMR. The presence of multiple isomers of the product will result in a complex spectrum with overlapping peaks, especially in the regions for the three propylene backbone protons. However, the methylene group on the terminal thioethers should be observed as singlets if the carboxylate group is not coordinated and a doublet of doublets (each proton unique) if it is coordinated to the Rh center.

Based on ^{1}HNMR and COSY analysis it is evident that multiple Rh-S4-(COOH)$_2$ species are present in each of the samples. 3,3,3-S4-(COOH)$_2$ is a symmetrical molecule. Each H is chemically equivalent to the corresponding H in the other half of the molecule (across the plane of symmetry). Thus the ^{1}HNMR spectraum of the uncomplexed 3,3,3-S4-(COOH)$_2$ chelate shows relatively few peaks (Figure 14). Upon complexation with Rh chloride, if there were only one *trans*-RhCl$_2$-S4-(COOH)$_2$ species produced with a single isomer, as previously believed, one would not expect any increase in the number of peaks observed, only a change in chemical shifts. ^{1}HNMR evaluation of the RhX$_2$-S4-(COOH)$_2$ complexes formed when prepared in aqueous solutions results in the appearance of many additional peaks. This is indicative of the presence of multiple RhX$_2$-S4-(COOH)$_2$ species (Figure 15), some of which may involve coordination to a pendant carboxylate group. Species with a coordinated carboxylate do not have a plane of symmetry. Each H on the molecule is chemically unique, and therefore a more comples spectrum (many more peaks) can be expected.

It is not possible to identify any species based on this ^{1}HNMR evaluation, but it is clear that multiple species are present. The multiple overlapping peaks can be explained by either formation of additional *trans*-RhX$_2$-S4-(COOH)$_2$ species (all differing in X$_2$ coordinated atoms) and/or the existence of more than one isomer of *trans*-RhX$_2$-S4-(COOH)$_2$.
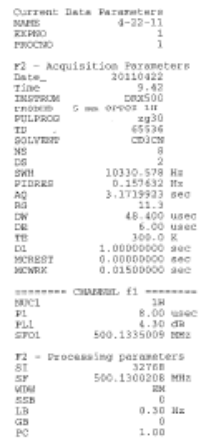
Figure 14: ^{1}HNMR of 3,3,3-S4-(COOH)$_2$ ligand.

sample 3 - H2O

Figure 15: ^{1}HNMR analysis of *trans*-[RhX$_2$-S4-(COOH)$_2$]$^+$ prepared in an aqueous solution.

Using ESI-MS several compounds were identified based on proposed structures as summarized in Figure 16 and Table 1. Samples employing reaction conditions (1) and (2) were very similar. Both were prepared in 50% ethanol/acetonitrile, sample (2) with the addition of excess NaCl. Under these conditions, $RhCl_2$-S4-$(COOH)_2$ (m/z = 544.91 Da, calc = 544.90 Da) (Figure 16 a) and $RhCl_2$-S4-(COOH)(COOEt) (m/z = 572.89 Da, calc = 572.93 Da) (Figure 16 b) were predominant and to a somewhat lesser extent RhCl-S4-(COOH)(COO-) where a pendant carboxylate group is coordinated to the metal center (m/z = 508.87 Da, calc =508.92 Da) (Figure 16 d) is also observed (Figures 17 and 18). The ethyl ester (Figure 16, b) is formed by acid catalyzed esterification in the presence of ethanol.

In sample (4) under aqueous conditions with excess NaCl the formation of $RhCl_2$-S4-$(COOH)_2$ (m/z = 544.91 Da, calc = 544.90 Da) (Figure 16 a)is dominant. However small amounts of RhCl-S4-(COOH)(COO-) (m/z = 508.87 Da, calc =508.92 Da) (Figure 16 d), $RhCl_2$-S4-(COOH)(COOEt) (m/z = 572.89 Da, calc = 572.93 Da) (Figure 16 b) and $RhCl_2$-S4-$(COOEt)_2$ (m/z = 600.91 Da, calc = 600.96 Da) (Figure 16 c) are also present (Figure 20).

The presence of all five species was observed in sample (3) prepared in aqueous conditions (25% water, 25% ethanol, 50% acetonitrile no salt). A significant yield of all three species $RhCl_2$-S4-$(COOEt)_2$ (m/z = 600.91 Da, calc = 600.96 Da) (Figure 16 c), RhCl-S4-(COOH)(COO-) (m/z = 508.87 Da, calc =508.92 Da) (Figure 16 d), and RhCl-S4-(COOEt)(COO-) (m/z = 536.90 Da, calc =536.95 Da) (Figure 16 e) was observed (Figure 19). This is the only sample in which the monochloro pendant carboxylate coordinated RhCl-S4-(COOEt)(COO-) species was observed with a pendant ethyl ester. We had hypothesized that a $Rh(OH)_2$-S4-$(COOH)_2$ species analogous to the observed $Rh(OH)_2$-S4-Diol may be present in aqueous solutions without NaCl, however this species was not observed.

There is a significant difference between sample (3), which was prepared in a solution of 25% water, 25% ethanol, 50% acetonitrile, and sample (4), which was prepared in the same solution with the

addition of 0.1 g NaCl. Without the presence of excess chloride ions in solution the pendant coordinated monochloride species RhCl-S4-(COOH)(COO-) and RhCl-S4-(COOEt)(COO-) are favored in addition to other species. With the presence of excess chloride ions in solution the dichloro species especially $RhCl_2$-S4-$(COOH)_2$ are more favored. The solvents used in radiolabeling are aqueous and it is likely that the concentration of NaCl present during radiolabeling is in between the conditions in samples (3) and (4). Therefore the addition of more NaCl might favor the production of a more dominate single $^{105}RhX_2$-S4-$(COOH)_2$ species.

The pendant ethyl esters on species (b), (c) and (e) (Figure 16) are formed by acid catalyzed Fischer esterification of the pendant carboxylic acid. An acidic solution is required to prevent the formation of $^{105}Rh(OH)_3$ and ethanol is required as a reducing agent. If neither acid nor ethanol can be eliminated, the formation of ethyl esters will continue to be a competing reaction for this formulation. The results of this study indicate that traditional radiolabeling techniques used for ^{105}Rh are not compatible with molecules that contain carboxylic acid groups.
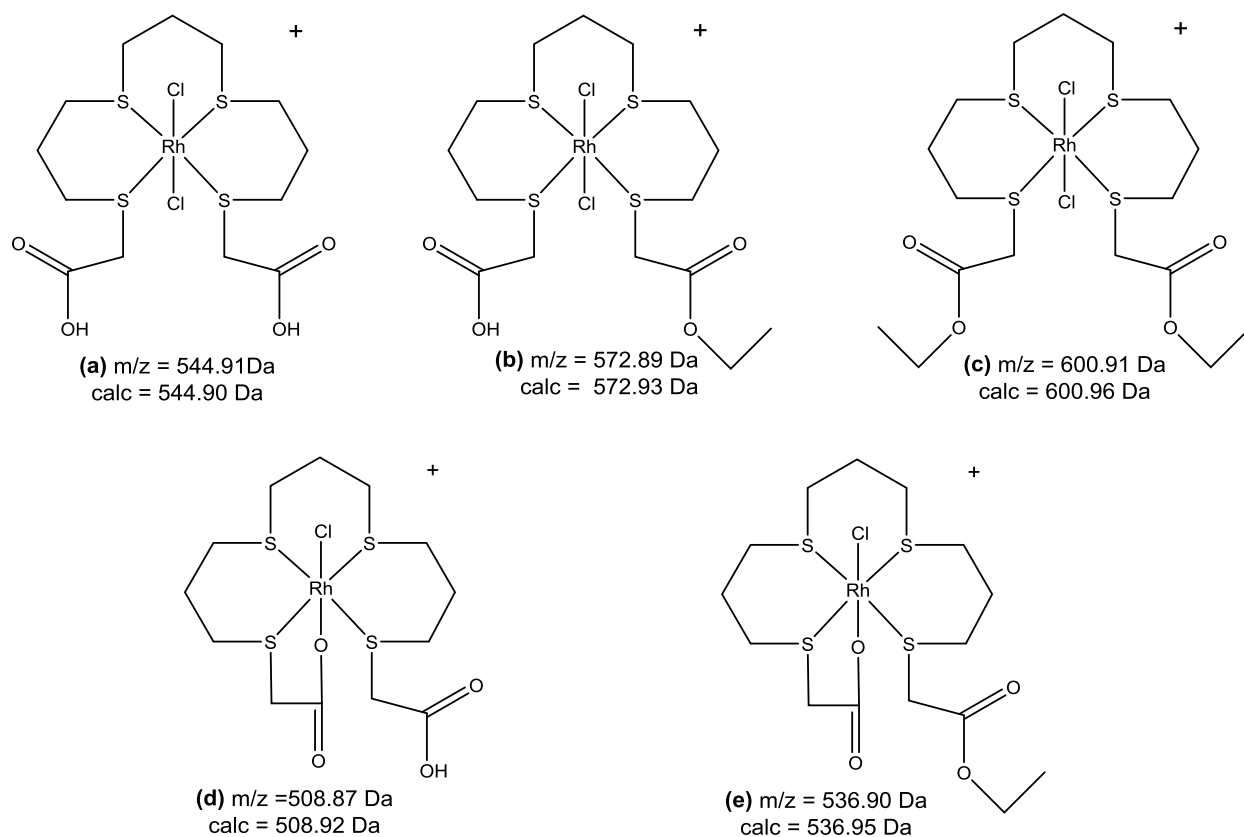
(a) m/z = 544.91Da
calc = 544.90 Da

(b) m/z = 572.89 Da
calc = 572.93 Da

(c) m/z = 600.91 Da
calc = 600.96 Da

(d) m/z =508.87 Da
calc = 508.92 Da

(e) m/z = 536.90 Da
calc = 536.95 Da

Figure 16: Rh-S4-(COOH)$_2$ species identified in samples 1-4.

Sample	Reaction Conditions	Products observed
1	50% ethanol/acetonitrile	**a**, **b**, d
2	50% ethanol/acetonitrile + NaCl	**a**, **b**, d
3	25% water, 25% ethanol, 50% acetonitrile	a, b, **c**, **d**, **e**
4	25% water, 25% ethanol, 50% acetonitrile + NaCl	**a**, b, c, d

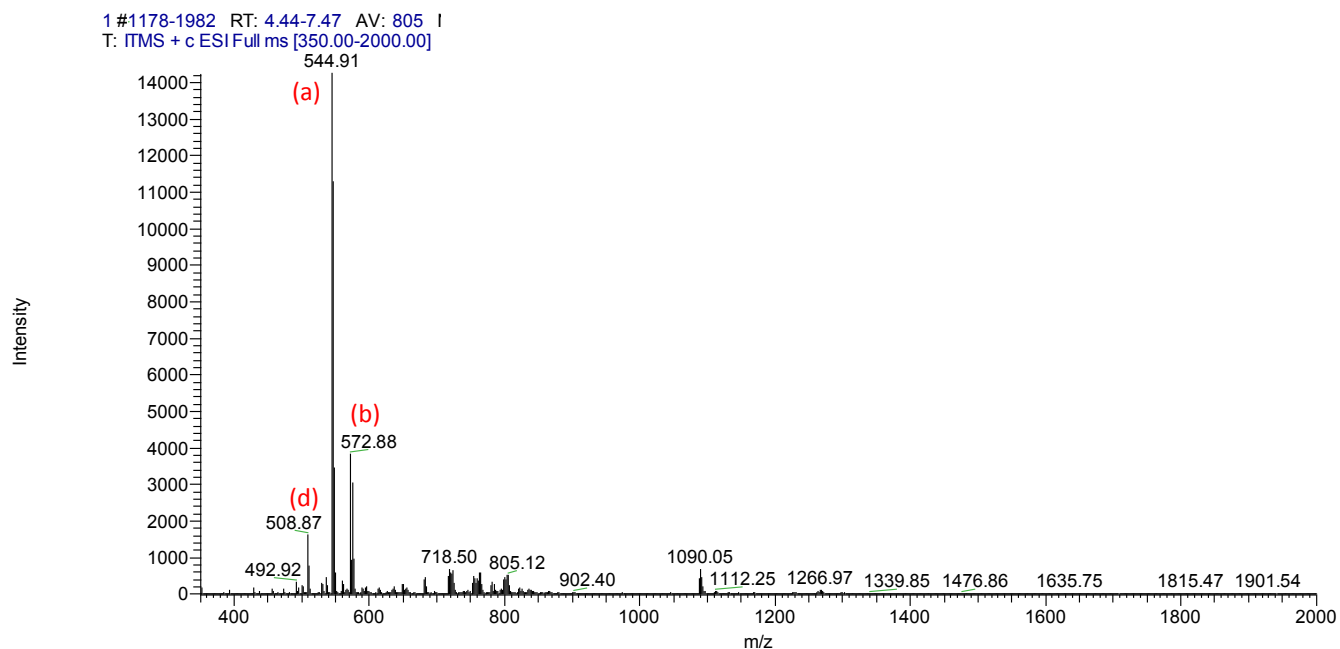**Table 1:** Products observed for various reaction conditions

Figure 17: Rh-S4-(COOH)$_2$ Sample (1) prepared in 50% ethanol/acetonitrile.
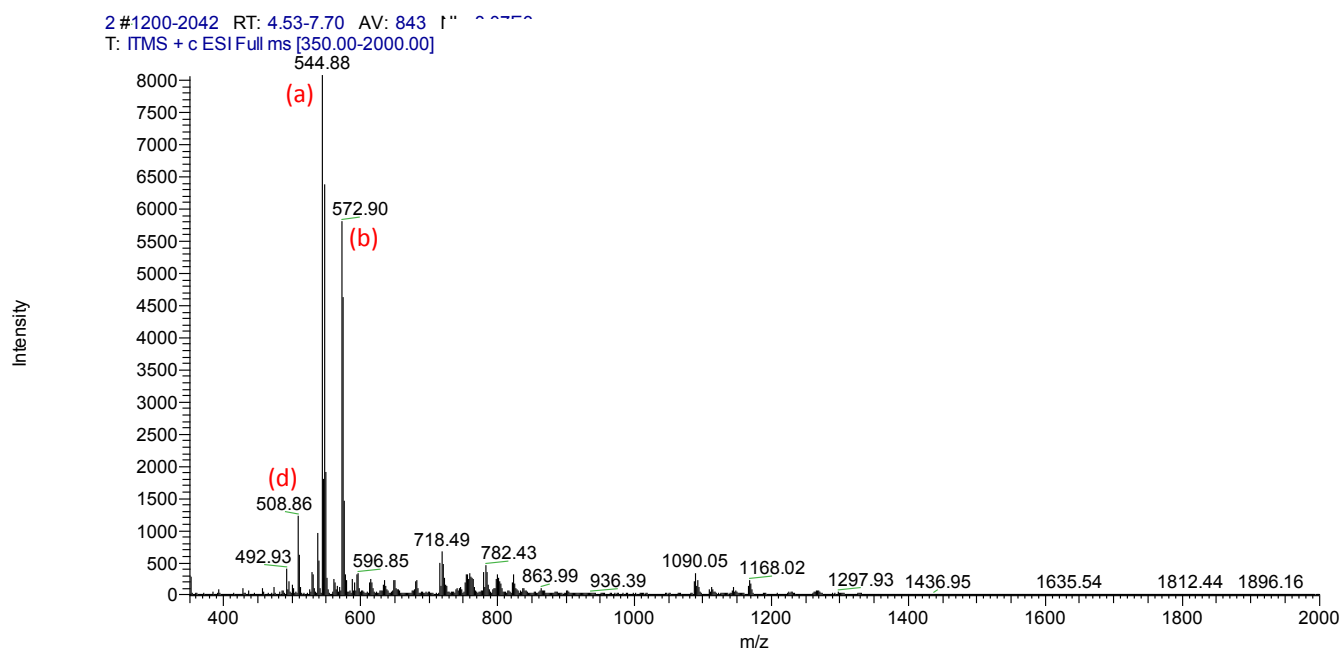
Figure 18: Rh-S4-(COOH)$_2$ Sample (2) prepared in 50% ethanol/acetonitrile + NaCl.
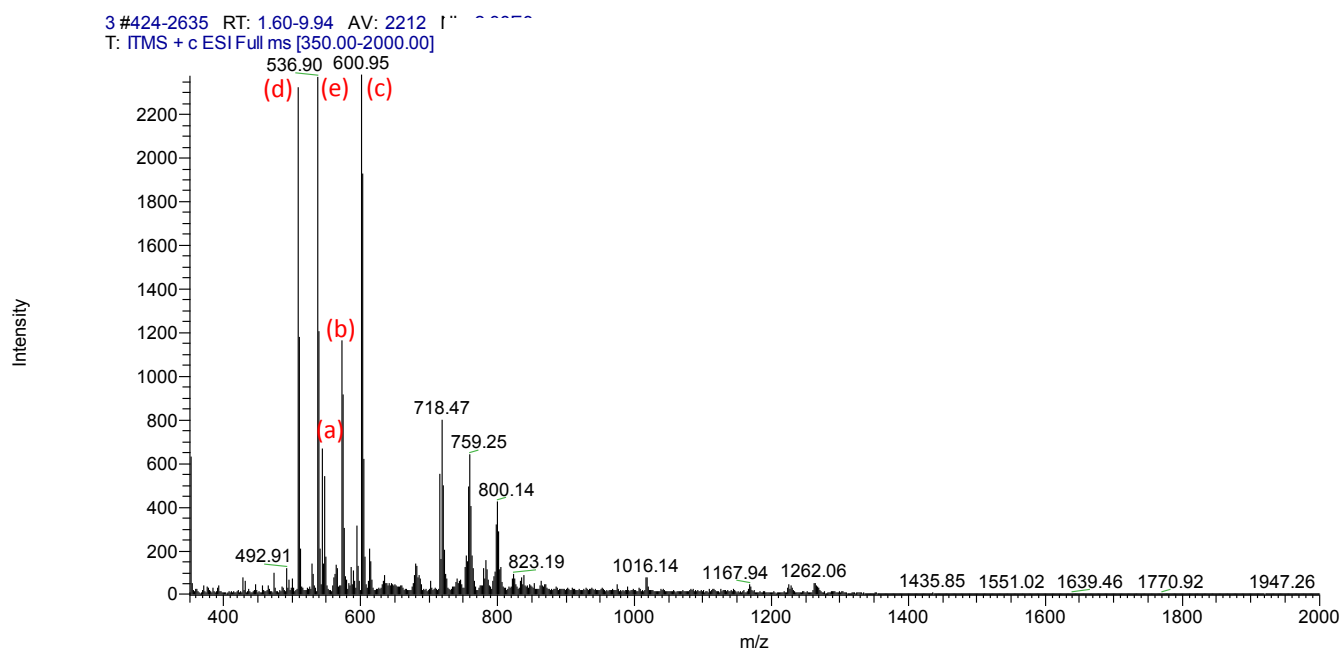
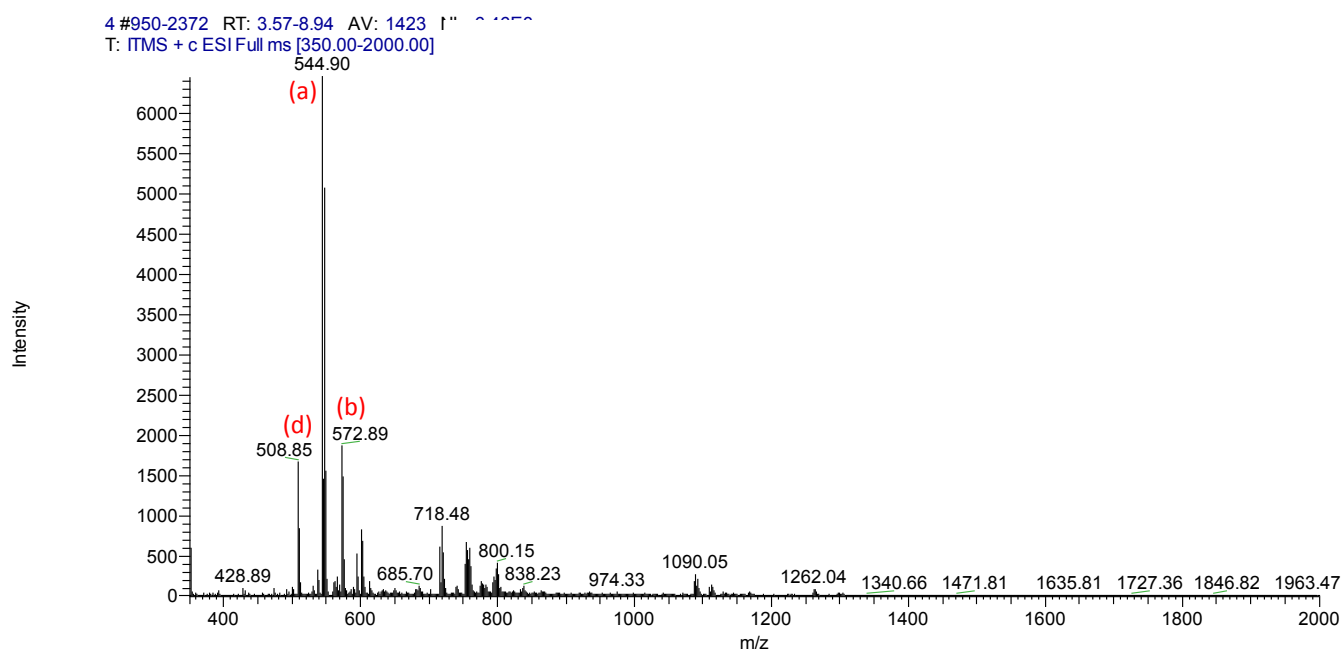**Figure 19:** Rh-S4-(COOH)$_2$ Sample (3) prepared in 25% water, 25% ethanol, 50% acetonitrile.

Figure 20: Rh-S4-(COOH)$_2$ Sample (4) prepared in 25% water, 25% ethanol, 50% acetonitrile + NaCl.

Specific Aim 5: Animal biodistribution studies

Animal biodistribution studies could not be carried out since a high yield of a single conformer of $[^{105}RhX_2\text{-}S4\text{-}8Aoc\text{-}BBN(7\text{-}14)NH_2]^+$ could not be obtained. Indeed recent studies indicate that formation of a single species cannot be formed with any chelate that contains a carboxylic acid group using traditional radiolabeling techniques for ^{105}Rh.

Specific Aim 6: Present and publish results

This work has been published in the form of a Ph.D. thesis and has also been prepared in paper form to be submitted for publication in the near future.

Training Program

This grant has provided me with many opportunities to develop my career. I have benefited from one on one mentoring with leaders in the field of Radiopharmaceutical Chemistry, Dr. Silvia Jurisson and Dr. Timothy Hoffman. I have attended symposia offered by the University of Missouri Chemistry Department and the Radiopharmaceutical Sciences Institute and benefited from intensive coursework in chemistry. I have had the opportunity to attend and present my research at the American Chemical Society meeting in 2012. I successfully completed my degree in May 2013, earning a Ph.D. in chemistry based on research supported by this grant. I have accepted a postdoctoral position with Dr. David Wilson at University of California San Francisco, where I plan to continue my work as a prostate cancer researcher focusing on the development of redox sensitive positron emission radiotracers.

Key Research Accomplishments:

- Synthesized a new rhodium tetrathioether molecule with high affinity for the BB2 receptor on human prostate cancer PC-3 cells
- Developed a new quantitative QC method for analyzing radiolabeling efficiency of rhodium-105

- Analyzed the effect of chloride ion concentration in labeling solutions of final radiolabled 105Rh tetrathioether products for cyclic (3,3,3,3-S4-Diol) and acyclic (3,3,3-S4-(COOH)2 chelate systems.

- Discovered that traditional rhodium-105 radiolabeling techniques are not compatible with molecules that contain a carboxylic acid group.

Reportable Outcomes:

Presentations

Carroll, V; Wycoff, D; Sieckman, G; Hoffman, T; Jurisson, S; "Synthesis of a [105]Rh tetrathioether bombesin molecule for prostate cancer therapy" 243rd National Meeting of the American Chemical Society: San Diego, CA, March 25 – 29, 2012

Manuscripts

Carroll, V; Demoin, D; Hoffman, T; Jurisson, S; *Inorganic Chemistry in Nuclear Imaging and Radiotherapy: Current and Future Directions.* Radiochimica Acta, 2012. **100:** p. 653 – 667

Carroll, V; *Development of a Rhodium Tetrathioether Bombesin Analogue and Investigation of Cyclic and Acyclic Ligand systems for [105]Rh(III).* Ph. D. Thesis, Department of Chemistry. 2013, University of Missouri. p. 94

Carroll, V; Wycoff, D; Sieckman, G; Gallazzi, F; Hoffman, T; Jurisson, S; *Synthesis of a Rh tetrathioether bombesin analogue and impact of labeling conditions on chelate systems with pendant carboxylate groups*, (in preparation)

Degrees Obtained

Ph.D. in Chemistry, University of Missouri, May 2013

Employment Opportunities Received

Postdoctoral Scholar, University of California San Francisco, Department of Radiology and Biomedical Imaging, Mentor: Dr. David Wilson

Conclusion:

We have successfully synthesized a novel rhodium bombesin conjugate, [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$. In vitro evaluation indicates high affinity for PC-3 human prostate cancer cells however low radiochemical yields of a single [^{105}Rh-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ species on the radiotracer scale may preclude its usefulness as a radiotheraputic agent. The pendant carboxylic acids were thought to be useful for maintaining reasonable hydrophilicity and thus clearance through the renal system. However, pendant carboxylic acids resulted in both complexes with a coordinated carboxylate and in esterification, the latter is particularly a problem at the radiotracer level where acidic ethanolic reaction conditions are needed. In

place of an acyclic bifunctional chelate (BFC) system with a pendant carboxylic acid, it is recommended

to change the direction of research in future studies to investigate cyclic BFC's or acyclic BFC's with a

methyl ester or methyl ether pendant group. This will prevent the formation of ethyl esters during

radiolabeling.

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2. Li, N., et al., *Biodistribution of model 105Rh-labeled tetradentate thiamacrocycles in rats.* Nuclear Medicine and Biology, 1997. **24**(1): p. 85-92.

3. Venkatesh, M., et al., *An Rh-105 complex of tetrathiacyclohexadecane diol with potential for formulating bifunctional chelates.* Nuclear Medicine and Biology, 1996. **23**(1): p. 33-40.

4. Goswami, N., *^{105}Rh(III) Complexes With Acyclid Tetrathioether Ligands: Potential Radiotherapeutic Agents*, in *Department of Chemistry*. 1996, University of Missouri. p. 160.

5. Goswami, N., et al., *Rhodium(III) Complexes with Acyclic Tetrathioether Ligands. Effects of Backbone Chain Length on the Conformation of the Rh(III) Complex.* Inorganic Chemistry, 1996. **35**(26): p. 7546-7555.

6. Goswami, N., et al., *Rhodium-105 tetrathioether complexes: radiochemistry and initial biological evaluation.* Nuclear Medicine and Biology, 1999. **26**(8): p. 951-957.